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Capsid protein gene and the type of host plant differentially modulate cell-to-cell movement of cowpea chlorotic mottle virus

A. L. N. Rao · B. Cooper

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Abstract A study was undertaken to measure the rate of coat protein (CP) independent cell-to-cell movement of *cowpea chlorotic mottle bromovirus* (CCMV) in three different host plants. A CCMV RNA3 variant in which the CP gene was substituted with enhanced green fluorescent protein (C3/ΔCP-EGFP) was coinoculated to three different host plants with transcripts of wild type RNAs 1 and 2. Comparative analysis of cell-to-cell movement monitored by the EGFP expression at various days post inoculation revealed that the rate of spread varied with the type of host species inoculated: fastest movement was observed in *Nicotiana benthamiana* while the rate spread was significantly slower in the natural host cowpea. When CP was expressed as EGFP fusion (C3/CP:EGFP) the rate of spread in *N. benthamiana* and *C. quinoa* was slower than that was observed in the absence of CP and remained subliminal in cowpea. Analysis of infection foci by confocal laser scanning microscope revealed that localization of CP:EGFP fusion was distinct in *N. benthamiana* and *C.quinoa* and accumulated as fluorescent inclusions at the cell periphery. Additional experiments involving coinoculation of either C3/ΔCP-EGFP or C3/CP:EGFP with heterologous *brome mosaic bromovirus* (BMV) genomic RNAs 1 and 2 revealed that, in addition to movement protein and CP, viral replicase also influences cell-to-cell spread. The significance of these results in relation to the mechanism of bromovirus movement is discussed.

A. L. N. Rao (✉)
Department of Plant Pathology, University of California, Riverside, CA 92521-0122, USA
e-mail: arao@ucr.edu

B. Cooper
Soybean Genomics and Improvement Laboratory, USDA-ARS, Beltsville, MD 20705, USA

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Introduction

Following replication in primary cells, most plant viruses are transported into neighboring nonvascular healthy cells through an active process that is often regulated by virus-encoded movement proteins (MP) [1–3]. In the absence of such cell-to-cell movement, the infection remains subliminal [4–6]. For some plant viruses, in addition to MP, coat protein (CP) is also necessary [7, 8]. In the CP-independent mechanism, MP alone mediates cell-to-cell movement by modifying the size exclusion limits of the plasmodesmata and transporting themselves and viral genomic RNA as ribonucleoprotein complexes. The cell-to-cell movement process of members of the *tobamovirus* and *dianthovirus* genera are CP-independent [2, 9] whereas that of members of the genera *Comovirus, Closterovirus, Cucumovirus, Bromovirus, Nepovirus, Potyvirus* and *Potexviruses* is CP-dependent [1, 5, 8, 10, 11]. In addition to MP and CP, in some viruses replicase genes are implicated in local and systemic infection [1, 12, 13]. However, not all RNA viruses share the requirement for CP to achieve long distance movement as exemplified for the members of the genera *tombusviruses* [14] and *hordeiviruses* [15].

The genomes of two members of the genus *Bromovirus*, namely *Cowpea chlorotic mottle virus* (CCMV) and *Brome mosaic virus* (BMV), are divided among three RNAs of messenger sense polarity [16]. Viral RNA replication is dependent on efficient interaction between two nonstructural proteins, 1a and 2a, encoded by monocistronic RNAs1 and 2 respectively [17]. The genomic RNA3 encodes a 5′

non-structural MP of 32 kDa and a 3′ CP of 19 kDa, which is synthesized via subgenomic RNA by internal initiation on negative strand RNA3 [16, 17]. The two gene products encoded by the dicistronic RNA3 are dispensable for viral replication, but are required for whole plant infection [5, 17].

Nicotiana benthamiana and *Chenopodium quinoa* are susceptible to BMV and CCMV infection. *N. benthamiana* is a symptomless host for BMV and CCMV [16]. In. *C. quinoa*, BMV induces chlorotic local lesions followed by systemic mottling whereas CCMV induces necrotic local lesions and upper uninoculated leaves remain uninfected [6]. Analysis of hybrid viruses constructed by exchanging CP genes between BMV and CCMV revealed that host range is not regulated by the CP [18]. Additional virus–host interaction studies revealed that MP gene is not only critical for cell-to-cell movement but also responsible for host specificity and modulation of symptom expression [19, 20]. Both MP and CP are required for cell-to-cell movement of monocot adapted BMV in *C. quinoa* and *N. benthamiana* [5, 6]. By contrast, CCMV variants devoid of the CP gene are competent for epidermal cell-to-cell movement but are defective in long distance spread in *N. benthamiana, C. quinoa* and cowpea [6]. However, Schneider et al. [21] reported that an encapsidation defective variant of CCMV was capable of delayed local and systemic spread in cowpea and *N. benthamiana*. Collectively the differential movement behavior between BMV and CCMV suggested that the requirement of CP for CCMV movement is relaxed compared to that of BMV. This study was undertaken to gain more insight as to the specific requirements and the form in which the CP is required (free or encapsidation competent) for cell-to-cell movement of CCMV in three different hosts (*C. quinoa*, cowpea and *N. benthamiana*). To this end, we have constructed a variant of CCMV RNA3 capable of expressing the enhanced green fluorescent protein (EGFP) reporter gene as a CP fusion (CP:EGFP) and quantitatively analyzed the relative rate of cell-to-cell movement and subcellular localization by confocal laser scanning microscopy (CLSM). In addition, we also examined the effect of heterologous replicase genes of BMV on CCMV cell-to-cell movement.

Materials and methods

Plasmid constructions

Full-length cDNA clones corresponding to the three genomic RNAs of CCMV, pTCC1TP1, pCC2TP2 and pCC3TP4, from which wild type (wt) infectious RNAs 1 (C1), 2(C2) and 3(C3), respectively, can be transcribed *in vitro*, have been described previously [22]. Similarly, plasmids pT7B1, pT7B2 and pT7B3 contain fill-length cDNA copies of the genomic RNAs 1(B1), 2(B2) and 3(B3), respectively [23]. The construction and characterization of a variant clone of CC3 in which the CP gene was replaced with that of EGFP (C3/ΔCP-EGFP) was previously described [6]. In order to fuse the sequence encoding the gene of EGFP to the carboxyl terminal region of CCMV CP, a *Sna*BI site was engineered immediately 5′ to the sequence coding for the CP stop codon by polymerase chain reaction (PCR) using a 5′ oligonucleotide primer and d(AGGCCACACTAGTGTAACGCTCTTCAGCGGGCA-TACGTACACCGGAGT; *Sna*BI site is underlined) and a 3′ oligonucleotide primer d(TGGCTGATTATGAAC-TAGTCGCGGCCGCT; *Spe*I site is underlined). The resulting PCR product was digested with *Hpa*I (located at position 1383) and *Spe*I (located at position 1953; Fig. 1) and subcloned into pCC3TP4, previously digested with the same restriction endonucleases, to yield pCC3/*Sna*BI. A PCR product encompassing the entire sequence of EGFP flanked by *Sma*I and *Spe*I restriction sites was amplified using a 5′ oligonucleotide primer d(CATGCTCAC-CCCGGGGGCGACCGG; *Sma*I site is underlined) and a 3′ oligonucleotide primer d(TGGCTOATTAT-GAACTAOTCGCGGCCGCT; *Spe*I site is underlined). The PCR product was digested with *Sma*I and *Spe*I and sub-cloned into *Sna*BI and *Spe*I digested pCC3/*Sna*BI, to yield pCC3/CP:EGFP (Fig. 1). The sequence surrounding the CP and EGFP fusion was verified by dideoxysequencing.

In vitro transcriptions and biological assays

Full-length and variant cDNA clones of CCMV and BMV were linearized with *Xba*I and *Bam*HI, respectively, and capped transcripts were synthesized *in vitro* with T7 RNA polymerase [22, 23]. Isolation and transfection of protoplasts from barley and *N. benthamiana*, extraction of total progeny RNA and their analysis by Northern hybridization using riboprobes of desired specificity were performed [24, 25]. For whole plant inoculations, *N. benthamiana, C. quinoa* and cowpea (cv. Black Eye) plants were kept in the dark for at least 18 h and mechanically inoculated [25]. Each experiment was repeated three to five times with independently synthesized in vitro transcripts. The inoculated plants were kept in the greenhouse at 25°C for observation.

Detection of EGFP in whole leaves

Leaves of *N. benthamiana, C. quinoa* and cowpea inoculated with EGFP constructs were illuminated with a hand

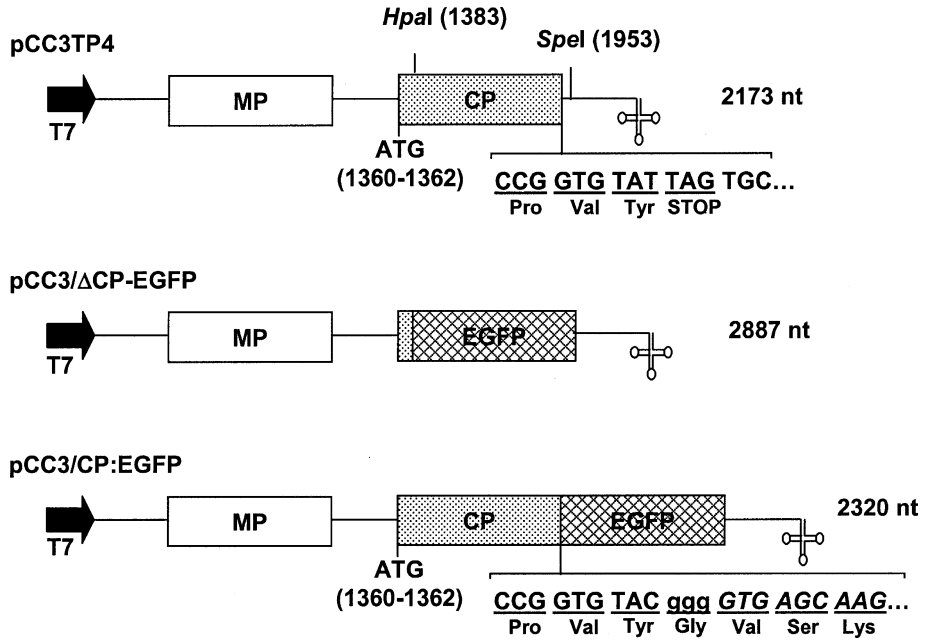

Fig. 1 Schematic representation of CCMV RNA3 variants. The characteristics of wt CCMV RNA3 (pCCTP4; 22) is shown with non coding sequences represented as single lines, movement protein (MP) as open box and CP as stippled box. The positions of selected restriction sites in pCCTP4 used to manipulate the CP gene are shown. The locations of start codon (ATG) and amino acid sequence surrounding the stop codon (TAG) are shown. In pCC3/CP:EGFP the entire EGEP gene (a portion of its amino acid sequence is shown in italicized capital letters) beginning with the second amino acid (Val) was fused to the last amino acid (Try) of CCMV MP gene (a portion of its amino acid sequence is shown in capital letters). These cloning manipulations introduced a foreign amino acid residue, Gly (shown in lower case letters and underlined), at the junction of CP and EGFP fusion. The characteristic features of pCC3/ΔCP-EGFP were described previously [6]. The arrow at the 5′ end represents the promoter for T7 RNA polymerase

held long wavelength UV light (366 nm) to detect fluorescent zones of infection. Whole leaves were also viewed under a Nikon Labophot microscope equipped with a super high pressure mercury lamp as a source for blue light (Nikon HB 10101AF), an epifluorescence attachment HFX-IIA and an FITC filter set XF23 (Omega Optical) containing a 488DF22 excitation filter, a 5OSDRLPO2 dichroic filter and a 535DF35 barrier filter. Photographs were taken with a Kodak Ektachrome 400 ASA slide film. Images were scanned from slides with a Nikon scanner (LS 1000) and graphically arranged using Adobe Photoshop 6.0 (Adobe Systems Inc., Mountainview, CA).

Confocal laser scanning microscopy

Confocal laser scanning micrographs were taken with an Olympus microscope (IX70). Focal planes were scanned with the 488 nm argon laser using a 550 nm (FVX-BA550RIF) barrier filter and 20×, 40× oil-free or 60× oil-immersion objectives. Optical sections were made at 1 μm intervals. Images were processed using the software (Fluoview 1.263) provided by the manufacturer and arranged using Adobe Photoshop 6.0.

Results

Movement of C3/ΔCP-EGFP is differentially regulated by the type of host plant

Previously it was observed that, unlike BMV, a CP deficient variant of CCMV RNA3 expressing free EGFP (C3/ΔCP-EGFP) was competent for movement between epidermal cells in *N. benthamiana, C. quinoa* and cowpea [6]. In the present study, the relative rate of cell-to-cell spread of C3/ΔCP-EGFP in each of these three hosts was monitored and quantitated by epifluorescence microscopy. These results are summarized in Table 1 and representative epifluorescence images are shown in Fig. 2 (Panel I). The relative cell-to-cell spread of C3/ΔCP-EGFP was clearly distinct in each host examined (Fig. 2, panel 1; Table 1). Among the three hosts, *N. benthamiana* was found to support most rapid cell-to-cell movement with time, followed by *C. quinoa* while cowpea being least supportive (Fig. 2, panel IA-F; Table 1). In *N. benthamiana* the earliest fluorescence, although weak, was detected at 8 h post inoculation (hpi) and confined to 3–4 fluorescent cells (data not shown). By 1-day post inoculation (dpi), several infection foci encompassing 50 fluorescent cells were

Table 1 Analysis of cell-to-cell movement of C1+C2+C3/ΔCP-EGFP

Host	DPI	No. of cells expressing EGFP per No. infection foci						
		1–3[a]	4–10	11–20	21–30	31–40	41–50	>100
N. benthamiana	1	2[b]	10	7	7	6	7	0
	2	0	6	10	14	18	41	33
	3	0	5	5	23	41	45	63
	5	0	3	3	16	53	71	83
	7	0	0	1	1	2	63	71
C.quinoa	1	17	24	16	13	1	0	0
	2	6	32	30	23	4	2	0
	3	3	100	76	23	5	1	0
	5	3	75	46	22	4	1	0
	7	2	70	41	20	3	1	0
Cowpea	1	3	15	7	0	0	0	0
	2	3	7	3	10	0	0	0
	3	3	2	0	0	0	0	0
	5	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0

[a]Number of green fluorescent cells per infection focus

[b]Average number of infection foci from four independent experiments

readily visible under the epifluorescence microscope (Fig. 2, panel IA; Table 1). These foci continued to expand with time and by 2 dpi, infection foci with more than 100 fluorescent cells were clearly observed. By 5 and 7 dpi, majority of infection foci contained more than 100 fluorescent cells and at this time point, foci containing less than 30 cells were rather limited (Fig. 2, panel 1A; Table 1). The expansion of foci expressing EGFP was ceased after 7 dpi. Irrespective of number of dpi, no fluorescence was visible to the naked eye when these leaves were examined with a hand-held UV light.

CCMV interacts in an incompatible manner with *C. quinoa* plants inducing characteristic necrotic local lesions [6, 18]. Although *C. quinoa* supported cell-to-cell movement of C3/ΔCP-EGFP, the spread was not as rapid as that was seen in *N. benthamiana* (Fig. 2, panel IC and D; Table 1). In *C. quinoa*, even after 5 dpi, majority of infection foci contained no more than 40 fluorescent cells (Table 1) and infection foci containing 50 fluorescent cells were rather limited. Unlike in *N. benthamiana*, no foci with 100 or more fluorescent cells were observed even at 7 dpi (Fig. 2; Table 1). The movement characteristics of C3/ΔCP-EGFP in cowpea are described below.

Localization of CP:EGFP in infected leaves of *N. benthamina* and *C. quinoa* is distinct

Although CP deficient variants of CCMV are competent for cell-to-cell movement (Fig. 2, Panel I; 6), our inability to visualize EGFP without the aid of a microscope suggested that initial spread was not efficient to be visualized by hand held UV light (Table 1). This restricted movement could be attributed either to the absence of the CP, which perhaps is required to overcome host resistance responses [17, 18] or required to promote efficient movement in a non-virion form (i.e. as CP-RNA complex). Although encapsidation competent CP may play an intrinsic role in the movement of BMV [5, 6, 26, 27], such a form is not obligatory for the movement of CCMV [21]. To verify whether efficient CCMV cell-to-cell movement requires encapsidation or if the virus is able to move as a CP:RNA complex (i.e. non-virion form), another variant of CCMV RNA3, referred to as C3/CP:EGFP, was used. In this construct, the entire sequence encoding the ORF of EGFP (except the start codon) was fused to the carboxyl terminal amino acid region of CCMV CP ORF, yielding pCC3/CP:EGFP (Fig. 1). These cloning manipulations introduced an additional amino acid at the junction of the fusion (Fig. 1). RNA transcripts synthesized in vitro from pCC3/CP:EGFP, designated as C3/CP:EGFP, were 714 nucleotides longer than wt CCMV RNA3 (Fig. 1). Upon infection, more than 80% of the protoplasts showed green fluorescence indicating that the subgenomic RNA4 derived from C3/CP:EGFP was capable of synthesizing a CP:EGFP fusion protein (data not shown). In bromoviruses, the N- terminus and C-terminus of the CP subunit extend in opposite directions from the base of the β-barrel and both termini are envisioned to play an essential role in virion assembly [28]. Furthermore reciprocal interactions between adjacent C-termini result in the formation of non-covalent CP dimer that is essential for initiating virion assembly in vivo and in vitro [28]. In vitro assembly studies performed with CCMV CP subunits having altered C-terminal region further accentuated the importance of this region in virion formation [28]. Accordingly we were also unable to recover any virions from leaves expressing the CP:EGFP fusion

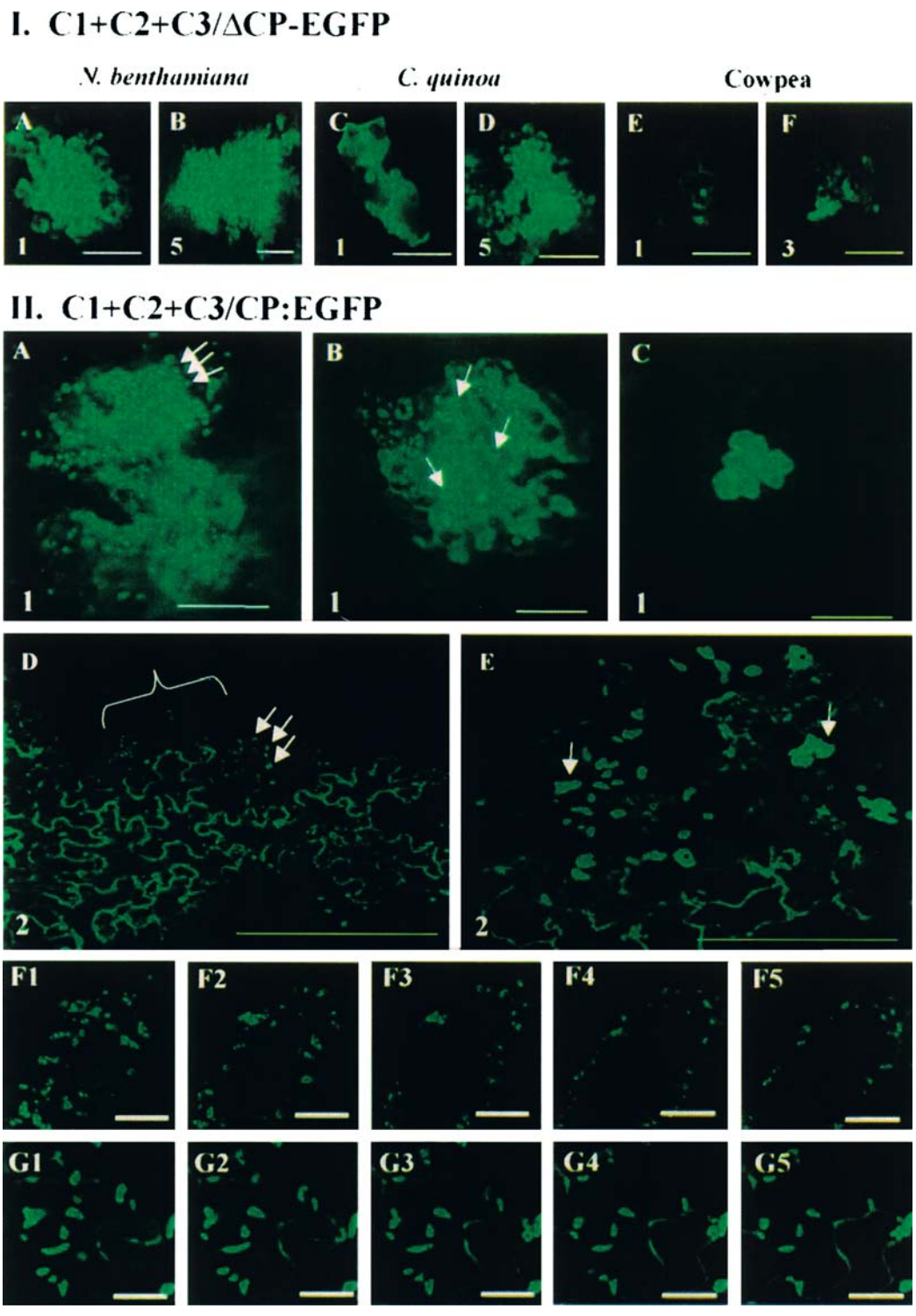

Fig. 2 Epifluorescence (panel I) and confocal laser scanning (panel I microscopic analysis of green fluorescence in cells infected with EGFP-expressing derivatives of CCMV RNA3. Panel I: Epifluorescence microscopic images demonstrating cell-to-cell movement of infection resulting from inoculations containing C1+C2+C3/ΔCP-EGFP in *N. benthamiana* (**a** and **b**), *C. quinoa* (**c** and **d**) and Cowpea leaves (**e** and **f**). Bar =100 μm for image shown in (**b**) and 50 μm for all other images. Panel II: Epifluorescence and CLSM analysis of green fluorescence in cells infected with CCMV RNA3 expressing EGFP when fused to CP. Epifluorescence photographic images demonstrate cell-to-cell movement of infection resulting from inoculations containing C1+C2+C3/CP:EGFP in *N. benthamiana* (**a**), *C. quinoa* (**b**) and cowpea leaves (**c**). Images shown in (**d**) and (**e**) represent CLSM of *N. benthamiana* and *C. quinoa* respectively. Bracket in (**d**) indicates infection front. Bar = 200 μm. Numbers in lower left hand corner of each image indicate days post inoculation (dpi). Images in (**f**) and (**g**) series represent CLSM consecutive 1 micron sections through an infection front in 1 dpi *C. quinoa* Leaves. For F series Bar = 20 μm and for (**g**) series Bar = 100 μm

protein since the C-terminal region of CCMV CP is blocked with the amino terminal region of EGFP (Fig. 1).

To monitor the rate of cell-to-cell movement and pattern of accumulation of the CP:EGFP fusion protein, *N. benthamiana, C. quinoa* and cowpea plants were inoculated with a mixture containing transcripts of C1+C2+C3/CP:EGFP and the inoculated leaves were observed by epifluorescence microscopy at different times post inoculation. Results are summarized in Table 2 and representative examples are shown in Fig. 2 (panel II, A–C). In *N. benthamiana*, the pattern of fluorescence induced by the fusion protein expressed from the subgenomic RNA of C3/CP:EGFP was clearly different from that of free EGFP expressed from the subgenomic RNA of C3/ΔCP-EGFP (compare panels IA and IIA in Fig. 2). One obvious difference between these two infections (i.e. C3/CP:EGFP vs C3/ΔCP-EGFP) was the relative intensity of fluorescence emitted from respective infection foci. In the case of foci expressing CP:EGFP fusion, the overall fluorescence was lower than that observed in foci expressing free EGFP. In addition, at a given time post inoculation, the rate of cell-to-cell-spread mediated by C3/CP:EGFP was much slower than that of C3/ΔCP-EGFP (compare data in Table 1 to Table 2). For example, at 1 dpi, majority of infection foci resulting from C3/CP:EGFP contained no more than 20 fluorescent cells and only few foci with 40 fluorescent cells were observed. By contrast at this time point several infection foci resulting from C3/ΔCP-EGFP have 50 fluorescent cells (Table 1, Table 2). For CP:EGFP even at 5 dpi, only a few infection foci contained more than 100 fluorescent cells (Table 2). In addition to rate of spread, interestingly, the pattern of fluorescence accumulation in each case was also different. Unlike uniform distribution of fluorescence throughout the cytoplasm and nucleus in cell infected C3/ΔCP-EGFP (Fig. 2, panel 1), the fluorescence in cells infected with C3/CP:EGFP was visible as bright fluorescent punctate bodies or inclusions (Fig. 2, panel II A). In contrast to *N. benthamiana*, cells of *C. quinoa* infected with C3/CP:EGFP contained large fluorescent inclusions (compare panel II A and B in Fig. 2). In each case the inclusions persisted up to 2 dpi and thereafter the intensity of the fluorescence diminished with time and by 6 dpi no fluorescent inclusion bodies were detected and the fluorescence was evenly distributed throughout the cytoplasm (data not shown).

Functional CCMV CP is required for efficient spread in cowpea

Cowpea is a natural host for CCMV and induces characteristic systemic chlorosis. Therefore, we anticipated that this host would promote rapid spread of C3/ΔCP-EGFP (i.e. free EGFP). However, unlike in *N benthamiana* and *C. quinoa* at 1 dpi, majority of infection foci induced in cowpea contained no more than 20 fluorescent cells (Table 1; Fig. 2, panel IF). By 2 dpi, few foci with 30 fluorescent cells were seen but this number did not increase but gradually faded with time (Table 1). At 3 dpi, the fluorescence emitted by EGFP in each infection foci was weaker than at 1 dpi and by 5–7 dpi no fluorescence could be seen (Table 1). The possible reasons for this unexpected fading EGFP with time are considered under the Discussion section. Since absence of CP reduced cell-to-cell movement in cowpea as compared to other two hosts (Table 1; Fig. 2, panel IA-F), we envisioned that CP even in non-encapsidated form might promote increased spread,

Table 2 Analysis of cell-to-cell movement of C1+C2+C3/CP:EGFP

Host	DPI	No. of cells expressing EGFP per No. infection foci						
		1–3[a]	4–10	11–20	21–30	31–40	41–50	>100
N. benthamiana	1	1[b]	26	11	4	2	0	0
	2	5	26	16	8	3	0	0
	3	1	11	11	13	2	2	0
	5	1	7	7	14	5	7	3
	7	1	1	2	13	5	6	1
C.quinoa	1	14	7	0	0	0	0	0
	2	5	28	10	0	0	0	0
	3	3	41	13	1	0	0	0
	5	2	6	18	7	2	1	0
	7	2	6	15	6	3	1	0
Cowpea	1	16	1	0	0	0	0	0
	2	11	0	0	0	0	0	0
	3	8	1	0	0	0	0	0
	5	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0

[a]Number of green fluorescent cells per infection focus

[b]Average number of infection foci from four independent experiments

as reported by Schneider et al. [21]. However, irrespective of time post inoculation of cowpea with C3/CP:EGFP (capable of expressing CP as EGFP fusion), majority of foci expressing green fluorescence contained 1–3 cells only (Fig. 2, panel IIC; Table 2). Unlike *N. benthamiana* and *C. quinoa*, no fluorescent inclusions were observed in these cells. Taken together, these observations suggested that the form in which CP is required to promote efficient spread in cowpea is clearly distinct from that of other two hosts.

Subcellular localization of CCMV CP:EGEP fusion by CLSM

The use of confocal laser scanning microscopy (CLSM) allowed us to limit the focal point to a single plane, thus increasing the resolution of images and reducing background fluorescence. A representative optical section of the infection front of CP:EGFP (Fig. 2, panel II D) confirmed our observation obtained by epifluorescence microscopy that fluorescent inclusions were prominent in newly infected cells (indicated by bracket in Fig. 2, panel II D). Representative confocal images of infected epidermal cells of *N. benthamiana* and *C. quinoa* showed fluorescent aggregate bodies at the front of the infection focus and as dissipated bodies in the cells behind the infection front (Fig. 2, panel II D and F). Magnification of the inclusions observed in *C. quinoa* cells revealed that the bodies were at the periphery of the cell, probably at the cell wall junctions (Fig. 2, panel I F). Optical sectioning from the upper part to the lower part of *N. benthamiana* and *C. quinoa* cells clearly confirmed that the bodies were localized at the periphery of the cell (Fig. 2, panel II F series and G series). We were also able to approximate the depth of the bodies through the cell. Since the optical distance of each section was 1 μm, it appeared that some inclusions reached sizes of 8 μm in depth revealing the extent of the CCMVCP accumulation in aggregates during infection (Fig. 2, panel II G series).

Effect of heterologous BMV replicase on cell-to-cell movement of C3/ΔCP-EGFP and C3/CP:EGEP

Unlike CCMV, the cell-to-cell movement of BMV is dependent on the expression of functional CP [5, 6, 27]. Detection of tubules containing virus-like particles in protoplasts transfected with BMV [26] suggested that BMV is likely to be transported between cells in fully assembled virion form, although there is no evidence yet that this mechanism prevails in plants. Since a pseudorecombinant virus assembled by mixing BMV RNAs 1 and 2 and CCMV RNA3 (i.e. B1 + B2 + C3) is biologically active [18, 22] and induces visible local necrotic lesions in *C. quinoa* [18], the effect of BMV replicase on the movement of C3/ΔCP-EGFP and C3/CP:EGFP was tested in *N. benthamiana* and *C. quinoa*. Irrespective of the time post infection and the host plant tested, inoculation of a mixture containing wild type transcripts of B1 and B2 and either C3/ΔCP-EGFP or C3/CP:EGFP resulted in subliminal infection foci characterized by having no more than 1–3 fluorescent cells (Table 3). Since the biological activity of an hybrid BMV RNA3 containing CCMV CP is indistinguishable from that of wt BMV (i.e. B1+B2+B3/CCMV CP; 18) and the observation that BMV RNAs 1 and 2 failed to support cell-to-cell spread of C3/CP:EGFP

Table 3 Effect of heterologous BMV replicase on cell to cell movement of C3/ΔCP-EGFP and C3/CP:EGFP

Host	DPI	No. of cells expressing EGFP per No. infection foci											
		B1+B2+C3/ΔCP-EGFP						B1+B2+C3/CP:EGFP					
		1–3[a]	4–10	11–20	21–30	31–40	41–50	1–3	4–10	11–20	21–30	31–40	41–50
N. benthamiana	1	1[b]	0	0	0	0	0	1	0	0	0	0	0
	2	5	0	0	0	0	0	4	0	0	0	0	0
	3	3	0	0	0	0	0	3	0	0	0	0	0
	5	3	0	0	0	0	0	3	0	0	0	0	0
	7	3	0	0	0	0	0	3	0	0	0	0	0
C.quinoa	1	0	0	0	0	0	0	0	0	0	0	0	0
	2	4	0	0	0	0	0	4	0	0	0	0	0
	3	2	0	0	0	0	0	2	0	0	0	0	0
	5	3	0	0	0	0	0	3	0	0	0	0	0
	7	3	0	0	0	0	0	3	0	0	0	0	0
Cowpea	1	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0
	3	1	0	0	0	0	0	0	0	0	0	0	0
	5	3	0	0	0	0	0	3	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0	0	0

[a]Number of green fluorescent cells per infection focus

[b]Average number of infection foci from four independent experiments

(Table 3), suggest a role for viral replicase in movement function. This conjecture is further supported by previous observations that mutations in viral replicase severely debilitated BMV movement [13].

Discussion

A previous study [6] involving the analysis of the cell-to-cell movement characteristics of a CP defective variant of CCMV RNA3 demonstrated that, unlike monocot adapted BMV, expression of a functional CP is not obligatory for initial cell-to-cell spared of CCMV in *N. benthamiana*, *C. quinoa* and cowpea. As a logical extension to these observations and to gain more insight into the mechanism and viral genes that regulate efficient cell-to-cell movement two CCMV RNA3 variants C3/ΔCP-EGFP and C3/CP:EGFP were engineered respectively to express EGFP in a free form and as a CP fusion. Experiments performed in this study with these two variants are designed to address two main issues: (i) to quantitatively analyze the relative rate of cell-to-cell spread and subcellular localization of CP in three different hosts; (ii) to elucidate the required form of CP to promote efficient cell-to-cell spread. Results of these experiments showed that (i) although CCMV CP is not obligatory for cell-to-cell movement, surprisingly in the natural host cowpea, expression of encapsidation-competent CP retains the ability to promote efficient CCMV spread; (ii) the rapid degradation of fluorescent signal resulting from the expression of EGFP as a CP fusion suggested that induction of post transcriptional gene silencing may affect virus movement; (iii) as observed with other viruses, replicase genes also affect cell-to-cell spread of CCMV.

The data presented here also revealed that the form of CP required for viral spread (together with MP) is clearly distinct for BMV and CCMV. This is evident after comparing the movement characteristics of C1+C2+C3/ΔCP-EGFP or C1+C2+C3/CP:EGFP to that of B1+B2+C3/CP:EGFP. In *N. benthamiana* and *C. quinoa*, common hosts for BMV and CCMV, cell-to-cell movement of CCMV occurred either in the absence of the CP (as in C3/ΔCP-EGFP) or in the presence of a non-encapsidating form of CP (as for C3/CP:EGFP). By contrast, BMV infections remained subliminal even when CCMV CP was supplied in a form incapable of forming virions (as in B1+B2+B3/CP:EGFP). This defective cell-to-cell movement manifested by B1+B2+B3/CP:EGFP is not due to the lack of BMV CP or the inability of the CCMV CP to interact appropriately with the BMV MP. This is exemplified by efficient cell-to-cell movement of pseudorecombinants constructed by exchanging wt RNAs 1 and 2 of BMV and RNA3 of CCMV or chimera harboring BMV MP and CCMV CP [18, 22]. Therefore, the fusion between the CP and the EGFP is inhibiting proper molecular interactions with components that are essential for viral spread or inhibiting the formation of virions which may be essential for the cell-to-cell spread of BMV but not completely for CCMV. Nevertheless, the inherent ability of CCMV CP to assist cellular spread of BMV suggest that encapsidation competent CCMV CP, while not essential for cellular spread, appears to be required to make the movement process more efficient.

This study also identified a distinctive pattern of CCMV CP:EGFP accumulating as aggregate bodies at the perimeters of *N. benthamiana* and *C. quinoa* cells (Fig. 2, panel II F and G series). It is noteworthy to compare the aggregates of CCMV CP to other EGFP viral protein fusions that have been studied in infected plant cells. For example, TMV MP, CMV MP and *groundnut rosette umbravirus* MP when fused to GFP, accumulated as aggregated bodies within plant cells whereas TMV MP:GFP aggregates dissipate over time similarly to CCMV CP:EGFP aggregates [11, 29, 30]. These MP aggregates appear near the cell walls of plants. Consequently they are thought be localized to plasmodesmata, the cellular channels that promote viral cell-to-cell spread. Since CCMV CP is not obligatory for cell-to-cell spread, the functionality of the CP association with plasmodesmata is not evident in the CCMV infection. However, CCMV CP retains an ability to facilitate cell-to-cell movement of BMV [18]. Thus, CCMV CP may aggregate in association with plasmodesmata, as implied by our confocal laser scanning microscope images. Such an association could be evolutionarily related to BMV helping CP to act in conjunction with the MP and assist cell-to-cell spread. The inclusions observed for CCMV CP:EGFP also appear to be similar in size to the inclusions formed by the potato virus X (PVX) potexvirus CP:GFP fusion [31]. In PVX, CP is required for cell-to-cell movement and it also associates with plasmodesmata [31]. Further investigation of CCMV CP localization at electron microscopic level will be necessary to understand the relationship between CCMV CP and cellular components.

Despite cowpea being the natural host, the movement of C3/ΔCP-EGFP or C3/CP:EGFP is relatively slower than other two hosts (Tables 1 and 2; Fig. 2, panels I F and II C). Of significant interest is the cessation of EGFP expression past 3 dpi (Tables 1 and 2). One possible explanation for this unexpected fading of EGFP in cowpea with time is the induction of post transcriptional gene silencing (PTGS; 32). Although suppressors of RNA silencing in BMV have not been identified, results of this study suggest that the RNA silencing pathways can vary depending on the host. For example, based on the EGFP expression by C3/ΔCP-EGFP or C3/CP:EGFP in all three

hosts it is surmised that encapsidation competent CP is required to function as a suppressor of RNA silencing in cowpea but not in the other two hosts. Experiments are in progress to analyze this conjecture.

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